Method for the purification of an N-terminal fragment of hepatocyte growth factor

The invention relates to a method for the purification of the N-terminal four kringle-containing fragment of hepatocyte growth factor (NK4).

Background of the Invention

Hepatocyte growth factor (HGF/SF) is a polypeptide identified and purified by Nakamura, T., et al., Biochem. Biophys. Res. Commun. 22 (1984) 1450-1459. It was further found that hepatocyte growth factor is identical to scatter factor (SF), Weidner, K.M., et al., Proc. Natl. Acad. Sci. USA 88 (1991) 7001-7005. HGF is a glycoprotein involved in the development of a number of cellular phenotypes including proliferation, mitogenesis, formation of branching tubules and, in the case of tumor cells, invasion and metastasis. For a status review, see Stuart, K.A., et al., Int. J. Exp. Pathol. 81 (2000) 17-30.

Both rat HGF and human HGF have been sequenced and cloned (Miyazawa, K. et al., Biochem. Biophys. Res. Comm. 163 (1989) 967-973; Nakamura, T., et al., Nature 342 (1989) 440-443; Seki, T., et al., Biochem. and Biophys. Res. Comm. 172 (1990) 321-327; Tashiro, K., et al., Proc. Natl. Acad. Sci. USA 87 (1990) 3200-3204; Okajima, A., et al., Eur. J. Biochem. 193 (1990) 375-381).

It was further found that an HGF/SF fragment, termed NK4, consisting of the N-terminal hairpin domain and the four kringle domains of HGF/SF has pharmacological properties that are completely different from those of HGF/SF, and is an antagonist to the influence of HGF/SF on the motility and the invasion of colon cancer cells, and is, in addition, an angiogenesis inhibitor that suppresses tumor growth and metastasis (Parr, C., et al., Int. J. Cancer 85 (2000) 563-570; Kuba, K., et al., Cancer Res. 60 (2000) 6737-6743; Date, K., et al., FEBS Lett. 420 (1997) 1-6; Date, K., et al., Oncogene 17 (1989) 3045-3054).

NK4 is prepared according to the state of the art (Date, K., et al., FEBS Lett. 420 (1997) 1-6) by recombinant expression of HGF cDNA in CHO cells and subsequent digestion with pancreatic elastase. Two other isoforms of HGF (NK1 and NK2) encoding the N-terminal domain and kringle 1, and the N-terminal domain and

kringles 1 and 2, respectively, were produced in E.coli via the inclusion body route (Stahl, S.J., Biochem. J. 326 (1997) 763-772). According to Stahl, naturation of NK1 or NK2 was performed in 100 mM TRIS/HCl pH 7.5 containing 2.5 M urea, 5 mM reduced glutathione (GSH) and 1 mM oxidized glutathione (GSSG). Purification was performed subsequently on a Superdex™ 75 column using also TRIS buffer. The use of TRIS buffer according to the state of the art during solubilization and naturation leads according to the investigations of the inventors to a considerable amount (of about 50%) of by-products which are identified by the inventors as consisting mainly of GSH-modified NK4.

Therefore this method is not useful for the recombinant production of NK4 in considerable amounts and sufficient purity (for therapeutic use).

Summary of the Invention

The invention provides a method for the production of NK4 by expression of a nucleic acid encoding said NK4 in a microbial host cell, isolation of inclusion bodies containing said NK4 in denatured form, solubilization of the inclusion bodies and naturation of the denatured NK4 in the presence of GSH and GSSG, characterized in that solubilization and naturation are performed at pH 7-9 in phosphate buffered solution.

It was surprisingly found that the use of potassium phosphate buffer in a pH range between 7 and 9, preferably between pH 8 and 9, leads to a considerable improvement in yield and purity of NK4.

Preferably NK4 is dialyzed after naturation with phosphate buffer pH 7-9 for at least 24 hours. Purificaton is performed preferably by hydrophobic interaction chromatography in the presence of phosphate buffer pH 7-9, whereby the use of butyl- or phenyl sepharose as chromatographic material is especially preferred.

Detailed Description of the Invention

Human HGF is a disulfide-linked heterodimer, which can be cleaved in an α-subunit of 463 amino acids and a β-subunit of 234 amino acids, by cleavage between amino acids R494 and V495. The N-terminus of the α-chain is preceded

by 31 amino acids started with a methionine group. This segment includes a signal sequence of 31 amino acids. The α-chain starts at amino acid 32 and contains four kringle domains. The so-called "hairpin domain" consists of amino acids 70-96. The kringle 1 domain consists of amino acids 128-206. The kringle 2 domain consists of amino acids 211-288, the kringle 3 domain consists of amino acids 305-383, and the kringle 4 domain consists of amino acids 391-469 of the α-chain, approximately.

NK4 according to the invention consist preferably of amino acid (aa) 32-494 or an N-terminal fragment thereof (always beginning with aa 32), the smallest fragment being aa 32-478. The length of NK4 can vary within this range as long as its biological properties are not affected. In addition there exist variations of these sequences, essentially not affecting the biological properties of NK4 (especially not affecting its activities antagonistic to HGF and its antiangiogenic activities), which variations are described, for example, in WO 93/23541. The activity of NK4 is measured by a scatter assay according to example 4.

NK4 can be produced recombinantly, either by the production of recombinant human HGF/SF and digestion with elastase (Date, K., FEBS Lett. 420 (1997) 1-6) or by recombinant expression of an NK4 encoding nucleic acid in appropriate host cells, as described below. NK4 glycoprotein has a molecular weight of about 57 kDa (52 kDa for the polypeptide part alone) and has the in vivo biological activity of causing inhibition of tumor growth, angiogenesis and/or metastasis.

The NK4 polypeptides can be produced by recombinant means in prokaryotes. For expression in prokaryotic host cells, the nucleic acid is integrated into a suitable expression vector, according to methods familiar to a person skilled in the art. Such an expression vector preferably contains a regulatable/inducible promoter. The recombinant vector is then introduced for the expression into a suitable host cell such as, e.g., E. coli and the transformed cell is cultured under conditions which allow expression of the heterologous gene. After fermentation inclusion bodies containing denatured NK4 are isolated.

Escherichia, Salmonella, Streptomyces or Bacillus are for example suitable as prokaryotic host organisms. For the production of NK4 polypeptides prokaryotes are transformed in the usual manner with the vector, which contains the DNA coding for NK4 and subsequently fermented in the usual manner. However

expression yield in E. coli using the original NK4 DNA sequence (GenBank M73239) is very low. Surprisingly it was found that modification of at least one of the codons of the DNA sequence encoding amino acid positions 33 to 36 (codon 33 encodes arginine, numbering according to M73239) results in an increase of expression yield of 20% polypeptide or more. Therefore, a further object of the invention is a method for the recombinant production of NK4 in prokaryotes by expression of a replicable expression vector containing DNA encoding NK4 characterized in that in said DNA at least one of the codons of amino acids selected from the group consisting of codons at positions 33, 34, 35 and 36 is modified from AGG to CGT (position 33), AAA to AAA (position 34), AGA to CGT (position 35), and/or AGA to CGT (position 36). It is further preferred that the codon for amino acid 32 is changed from encoding Gln to encoding Ser in order to improve splitting off N-terminal arginine.

Inclusion bodies are found in the cytoplasm as the gene to be expressed does not contain a signal sequence. These inclusion bodies are separated from other cell components, for example by centrifugation after cell lysis.

The inclusion bodies were solubilized by adding a denaturing agent like 6 M guanidinium hydrochloride or 8 M urea at pH 7-9 in phosphate buffer (preferably in a concentration of 0.1 – 1.0 M, e.g. 0.4 M) preferably in the presence of DTT (dithio-1,4-threitol). The solubilisate is diluted in phosphate buffer pH 7-9 in the presence of GSH/GSSG (preferably 2-20 mM glutathion) and a denaturing agent in a non denaturing concentration (e.g. 2M guanidinium hydrochloride or 4 M urea) or preferably instead of guanidinium hydrochloride or urea, arginine in a concentration of about 0.3 to 1.0 M, preferably in a concentration of about 0.7 M. Renaturation is performed preferably at a temperature of about 4°C and for about 48 to160 hours.

After naturation is terminated the solution was dialyzed preferably against phosphate buffer pH 7-9 (preferably in a concentration of 0.1 – 1.0 M, e.g. 0.3 M) for at least 24 hours, preferably for 24 – 120 hours.

NK4 polypeptide or fragments thereof can be purified after recombinant production and naturation of the water insoluble denatured polypeptide (inclusion bodies) according to the method of the invention preferably by chromatographic

methods, e.g. by affinity chromatography, hydrophobic interaction chromatography, immunoprecipitation, gel filtration, ion exchange chromatography, chromatofocussing, isoelectric focussing, selective precipitation, electrophoresis, or the like. It is preferred to purify NK4 polypeptides by hydrophobic interaction chromatography, preferably at pH 7-9, in the presence of phosphate buffer and/or preferably by the use of butyl- or phenyl sepharose.

According to the method of the invention, only a minor amount of the NK4 polypeptides is modified by the formation of GSH adducts. Of the total amount of NK4 polypeptides, i.e. the amount of the inclusion bodies separated from other cell components (corresponding to 100%), the amount of GSH-modified NK4 is between 0% and 50%, preferably between 0% and 35%, and more preferably between 0% and 20%.

The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

<u>Description of the Figures</u>

Figure 1	Renaturation kinetics, heparin column, detection at 280 nm
Figure 2	Renaturation efficacies

<u>Description of the Sequences</u>

SEQ ID NO:1	DNA coding for NK4
SEQ ID NO:2	Polypetide sequence of NK4

<u>Example 1</u>

Recombinant expression of NK4

The NK4 domain from amino acid position 32 to 478 of HGF was used for cloning and recombinant expression in Escherichia coli. The original DNA sequence used

as source of DNA was described (database identifier "gb:M73239"). PCR was performed in order to amplify and concurrently modify the DNA coding for NK4 (Seq ID No:1). All methods were performed under standard conditions.

In comparison to the original DNA sequence of NK4, the following modifications were introduced:

- Elimination of the eukaryotic signal peptide sequence and fusion of the ATG start codon next to amino acid position 32 of NK4
- exchange of amino acid position 32 from Gln to Ser in order to improve homogeneity of the protein product (Met-free)
- modification of the DNA sequence of the codons of amino acids at position 33 (AGG to CGT), 35 (AGA to CGT), and 36 (AGA to CGT) in order to improve gene expression in E.coli.
- modification of the DNA sequence of codons at position 477 (ATA to ATC) and 478 (GTC to GTT) in order to facilitate insertion of PCR product into the vector
- introduction of two translational stop codons at positions 479 (TAA) and 480 (TGA), in order to stop the translation at a position equivalent to the end of NK4 protein domain.

The PCR-amplified DNA fragment was treated with restriction endonucleases NdeI and BanII and was ligated to the modified pQE vector (Qiagen) (elimination of His-tag as well as DHFR coding region), which was appropriately treated with NdeI and BanII. The elements of expression plasmid pQE-NK4-Ser (Plasmid size 4447 bp) are T5 promotor/lac operator element, NK4 coding region, lambda to transcriptional termination region, *rrn*B T1 transcriptional termination region, ColE1 origin of replication and β-lactamase coding sequence.

The ligation reaction was used to transform E.coli competent cells, e.g. E.coli strain C600 harbouring expression helper plasmid pUBS520 (Brinkmann, U., et al., Gene 85 (1989) 109-114). E.coli colonies were isolated and were characterized with respect to restriction and sequence analysis of their plasmids. The selection of clones was carried out by analysis of the NK4 protein content after cultivation of recombinant cells in LB medium in the presence of appropriate antibiotics and after induction of the gene expression by addition of IPTG (1 mM). The protein

pattern of cell lysates were compared by PAGE. The recombinant E.coli clone showing the highest proportion of NK4 protein was selected for the production process. Fermentation was performed under standard conditions and inclusion bodies were isolated.

Example 2

Solubilization and naturation using the optimized conditions.

Inclusion bodies were dissolved over night in a buffer containing 6 M guanidinium hydrochloride, 0.1 M potassium phosphate pH 8.5 (by titration with 10 M KOH), 1 mM EDTA, 0.01 mM DTT. The concentration of the dissolved protein was determined by Biuret assay and finally adjusted to a concentration of 25 mg total protein / ml at room temperature .

This NK4-solubilisate was diluted to a concentration of 0.4 mg / ml in a buffer containing 0.7 M arginine, 0.1 M potassium phosphate pH 8.5 (by titration with conc. HCl), 10 mM GSH, 5 mM GSSG and 1 mM EDTA. This renaturation assay was incubated between 2 and 8 days at 4°C. The renaturation efficacy was measured by analytical affinity chromatgraphy using an 1 ml Heparin Sepharose column (renaturation kinetics see Fig.1).

Buffer conditions:
Buffer A: 50 mM Tris pH 8.0
Buffer B: 50 mM Tris pH 8.0, 2 M NaCl

Gradient:	5-25%	buffer B, 2 column volumes
	25-60%	buffer B, 16 column volumes
	60-100%	buffer B, 0.7 column volumes
	100%	buffer B, 2 column volumes

After obtaining the maximal renaturation efficacy, the renaturation assay of 15 l volume was concentrated to 3 l using a tangential flow filtration unit (MW cut off: 10 kDa, Sartorius). It was subsequently dialyzed against 3 times 50 l buffer containing 0.3 M potassium phosphate at pH 8.0 for at least 3 x 24 hours, optimally for 5 days in total.

Purification was performed by heparin-sepharose chromatography (conditions see above). To the eluted material 1 M ammonium sulfate in 0.1 M potassium phosphate pH 8.0 was added and incubated at 4°C overnight. The sample was centrifuged and the supernatant was loaded on a phenyl sepharose column (150 ml). The column was washed with 1 column volume 1 M ammonium sulfate in 50 mM potassium phosphate pH 8.0.

Elution conditions:
Buffer A: 1 M ammonium sulfate, 50 mM potassium phosphate pH 8.0
Buffer B: 50 mM potassium phosphate pH 8.0, 40 % ethylene glycol
0-100 % buffer B, 20 column volumes

Example 3

Comparison of naturation using potassium phosphate and Tris

Renaturation conditions were analyzed using potassium phosphate or TRIS at pH 7.5 and pH 8.5 (both titrated with conc. HCl) as buffering reagents. The solubilization and renaturation conditions were as described in example 2, but with 0.1 M TRIS or 0.1 M potassium phosphate in the renaturation buffer. The dialysis was also performed as described in example 2, but in 0.1 M TRIS or 0.1 M potassium phosphate. Potassium phosphate buffer (K-P) led to significantly higher renaturation yields as TRIS buffer, measured as amount of active NK4 by scatter assay (see Fig. 2).

Example 4

Determination of activity

a) Scatter assay

MDCK cells were subconfluently grown in tissue culture plates. Cells were treated with HGF (10 ng/ml) or with combinations of HGF and NK4. In these experiments the HGF-induced cell scattering was inhibited by the addition of a 10 to 1000-fold molar excess of NK4 at least for 90% and more, showing the functional activity.

b) Proliferation assay

Inhibition of the mitogenic activity of HGF by NK4 was determined by measuring DNA synthesis of adult rat hepatocytes in primary culture as described in Nakamura, T., et al., Nature 342 (1989) 440-443. In these experiments the HGF-induced cell proliferation was inhibited by the addition of a 10 to 1000-fold molar excess of NK4 at least for 90% and more, showing the functional activity.

c) Invasion assay

In this assay the invasive potential of tumor cells is analyzed. The assay was done essentially as decribed in Albini, A., et al., Cancer Res. 47 (1987) 3239-3245, using HT115 cells. Again, HGF-induced (10 ng/ml) cell invasion could be inhibited by a 10 to 1000-fold molar excess of NK4 at least for 90% and more, showing the functional activity.

Example 5

Activity in vivo

Model:	Lewis Lung Carcinoma nude mouse tumor model 1 x 10^6 lewis lung carcinoma cells were s.c. implanted into male nude mice (BALB/c nu/nu).
Treatment:	After 4 days, one application daily of pegylated NK4 over a period of 2-4 weeks
Dose:	1000 µg/mouse/day 300 µg/mouse/day 100 µg/mouse/day placebo
Result:	Treatment with NK4 shows a dose dependent suppression of primary tumor growth and metastasis, whereas no effect is seen in placebo treated groups.

List of References

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